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*ABERRANT HETEROZYGOTES IN ESCHERICHIA COLI**

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A mechanism of genetic recombination has been indicated in experiments on *Escherichia coli*, strain K-12.[1] A synthetic agar medium was used as a selective sieve to isolate occasional prototroph recombinants which appear in mixed cultures of complementary biochemical mutants. Later, additional genetic factors were introduced, including fermentation and virus-resistance mutations, and these factors were found to segregate in characteristic ratios, suggesting linkage. All the cells in a given prototroph colony showed the same combination of characters and were stable on further cultivation. Therefore, it was inferred that segregation had occurred before the initiation of the colony, and that the postulated zygote had a very short life, probably a single cell generation. The life cycle would resemble the ascomycete's, in which haploid nuclei fuse to form a transient diploid zygote which undergoes meiosis without any intervening mitoses.

Exceptions to this rule have now been found in the form of unstable prototroph cultures which continually segregate out various recombination types so as to suggest that they are heterozygous and diploid. But abnormalities in the segregation ratios suggest some sort of chromosomal aberration whose nature has not yet been proved.

Material and Methods.—The cultural and crossing techniques used earlier have generally been followed. Crosses were also made by spreading mixtures of cells on the surface of synthetic medium[2] to which carbohydrate and an eosin-methylene blue indicator are added. This medium, EMS-, classifies prototrophs as soon as they appear for fermentation of the sugar used. Other media used include EMB- agar, in which the indicator is incorporated in a sugar peptone base. The genetic factors involved in this study are summarized in table 1.

TABLE 1

SUMMARY OF MUTANTS

LOCUS	"TYPE" ALLELE	MUTANT ALLELE	CHARACTER OF MUTANT
B	+	−	Needs biotin for growth
M	+	−	Needs methionine
T	+	−	Needs threonine
L	+	−	Needs leucine
B_1	+	−	Needs thiamin
Lac	+	−	Unable to ferment lactose
Mal	+	−	Unable to ferment maltose
Gal	+	−	Unable to ferment galactose
Ara	+	−	Unable to ferment L-arabinose
Xyl	+	−	Unable to ferment D-xylose
Mtl	+	−	Unable to ferment mannitol
V_1	*s*	*r*	Resistant to phages T1, T5
V_{1c}	*s*	*r*	Resistant to T1; sensitive to T5
"Het"	..	..	Persistent heterozygote factor

Crosses Involving "Het."—The first unstable prototroph to be discovered, *"H-1,"* arose in a cross to test the allelism of the virus resistance factors V_1 and V_{1c}. The parents were B − M − Lac + V_{1c}^r and T − L − B_1 − Lac − V_1^r. Among 200 prototrophs picked and tested on synthetic agar for sensitivity to T1, only one was an apparent crossover showing a full sensitive reaction. However, when this unique culture was streaked out on EMB − lactose, it gave rise not to typical dark Lac+ or light Lac− colonies, but to highly variegated colonies with intermingled sectors of light and dark cells. The variegated reaction will be referred to as Lac*v*.

When Lac*v* colonies were streaked out again on EMB − lactose, pure Lac−, pure Lac+ and a few Lac*v* colonies were seen. But when the pure − and + colonies were inoculated on EMS − lactose they failed to grow, showing that they were nutritionally deficient. Furthermore, although they had been derived from a virus-sensitive culture, most of the purified "segregants" were resistant to T1 when tested on EMB − lactose. On the other hand, Lac*v* colonies gave rise to normal appearing Lac+ on

EMS—lactose, which, as before were sensitive to T1. Taken back to EMB—lactose, they showed the Lac*v* result of splitting off pure — and + types. When single colonies were streaked out in series for 15 transfers on EMS—lactose, they retained their normal Lac+ appearance, but throughout the experiment they continued to segregate when brought on EMB—lactose, which is a nutritionally "complete" medium. To summarize, *H-1* was an unstable prototroph culture which was apparently Lac+ and T1-sensitive so long as it was maintained on a synthetic medium, but which segregated nutritionally deficient, Lac— and T1-resistants on a "complete" medium.

At this point, three alternatives had to be distinguished. The segregating entities might be (*a*) associations of intact cells of the original parental mutants collaborating by syntrophism to allow growth on synthetic medium; or (*b*) heterokaryons comprising intact nuclei of the parents in the same cell; or finally (*c*) diploid heterozygotes. The phage sensitivity of *H-1*, contrasted to the resistance of the parents, render (*a*) unlikely, but the segregation might still be from a heterokaryon or a heterozygote. To test these alternatives, it was thought that a heterokaryon should split out each of the two parental genotypes, but no additional combinations. On the other hand, segregation from a heterozygote should sometimes be accompanied by crossing over, and the formation of new combinations of the parental characters.

One hundred thirty-five Lac*v* colonies were separately streaked out on EMB—lactose, and a single pure Lac+ and Lac— segregant isolated from each of the 135 streakings, to insure that each of the cultures tested was an independent segregant. Among the 135 Lac— tested, there were 121 $T5^r$ (parentals) and 14 $T5^s$ (exchanges); the Lac+ included 133 $T5^s$ (parentals) and 2 $T5^r$ (exchanges). Therefore, out of 270 tests there were 16, or 6%, new combinations. Therefore, crossing over occurs during the segregation of *H-1*. This is compatible only with the hypothesis of segregation from a heterozygote.

The following remarks should not obscure this major conclusion. The amount of exchange in the Lac— and Lac+ series is not the same ($\chi^2_1 = 9$). The crossovers in the two series came from different Lac*v* colonies, but reciprocality cannot be demanded if many different segregations occur during the growth of a single Lac*v* colony. Even the higher value of $^{14}/_{135}$ or 10% for exchanges between Lac and V_1 in the Lac— series is lower than the 38% derived in previous studies on prototroph recombinants. Finally, it was very obvious that the Lac— segregants far outnumbered the Lac+. If anything, selective pressures on EMB—lactose seem to favor Lac+, so this finding was most unexpected.

Owing to technical difficulties, tests on nutritional segregations have been less extensive. It proved to be especially difficult to score for the biotin requirement in the presence of amino acids, probably due to partial replacement. The classification of the thiamin requirement (B_1-) also requires fastidious attention to clean chemicals and glassware. Most of the Lac+T5^s segregants are the parental B−M−; Lac−T5^r are usually T−L−B_1−. However, prototrophs and the multiple mutant combinations M−T−L− and M−T−L−B_1− have been encountered. These combinations could not be obtained by previous methods, which necessarily isolated prototrophs.

Unstable heterozygotes like *H-1* had not been encountered before, although many hundreds of Lac+ prototrophs from crosses heterozygous for Lac had been streaked out on EMB−lactose. The persistence of *H-1* as a diploid might be a non-genetic accident or perhaps an effect of a spontaneous life cycle mutation. If so, it would be possible to set up crosses to yield diploids heterozygous for additional markers.

Three segregants from *H-1* have been used in crosses to test for the production of persistent heterozygotes. (*A*) (W-466) was B−M−Lac_1−V_1^s; (*B*) (W-477) T−L−B_1−Lac_1−V_1^r and (*C*) (W-478) B−M−Lac+V_1^s. These cultures were crossed on EMS−lactose with appropriate, "standard" complementary stocks (B−M− or T−L−B_1−; Lac− or Lac+) of independent origin. Lac+ prototrophy were streaked out on EMB−lactose, and Lac*v* colonies looked for. In each of these crosses, about 5% of the Lac+ prototrophs were heterozygous. Crosses of *A* × *C* gave the same result. If "*Het*" is located on a chromosome, it is effective whether present in one or both parents.

In further studies still in progress, *C* has been crossed with multiple fermentation mutant stocks, T−L−B_1−Lac_1−Mal−Gal−Ara−Xyl−, and some also Mtl−. Such stocks were developed by irradiations on EMB− media, in sequence. As before, about 5 to 10% of the + prototrophs isolated from EMS lactose or xylose plates are heterozygous. However, these cultures are not uniformly heterozygous for all the factors in which the parents differed. It is especially notable that in over a hundred heterozygotes obtained in this way, Mal has never been heterozygous. Usually, the cultures are pure Mal−, sometimes Mal+. Xyl*v* cultures are equally often Lac*v* or pure for Lac, as well as the converse.

It remains to be decided whether the "pure" characters are homozygous or hemizygous. The Mal locus which, as mentioned, has always been "pure" is probably hemizygous, as shown by reversion studies. If the cultures were homozygous, Mal− would be twice represented, i.e., Mal−/Mal−. Reverse mutations of one of these genes should lead to heterozygosity at the locus. Preliminary tests on reversions for Mal in these stocks have invariably given pure Mal+ cultures, although they still segregated for other factors. This suggests that Mal is represented only once, that it is hemizygous, and, therefore, that there is deficiency for a region including this locus. This tentative conclusion is in accord with the aberrant segregation of Lac (excess of Lac−) observed in *H-1*. The hypothetical deficiency would be lethal in a haploid, and lead to the loss of segregants carrying the alleles linked to it.

Some quantitative studies of segregation and crossing over have been carried out on these complex heterozygotes. *H-72* segregates both for lactose and xylose fermentation. Segregation was permitted to occur "enmasse" in heavy cultures in nutrient broth, the cultures diluted and plated on EMB− xylose and EMB−lactose. Of 895 colonies on EMB−xylose, 19 were still Xyl*v*; 815 Xyl− and 61 Xyl+. The Xyl+ were all Lac−. The segregation ratio is 815−:61+ or more than 13:1. Similarly, of 753 colonies on EMB−lactose, 23 were still Lac*v*; 654 Lac− and 76 Lac+ (all of which were Xyl−). Thus, no Lac+Xyl+ segregants were observed in this sample. The other classes are computed to be Lac−Xyl− 83%, Lac−Xyl+ 7% and Lac+Xyl− 10%. These figures might be accounted for by mapping:

Xyl+ *7.7* Lethal *11.2* Lac+/Xyl−...Lac−.

To justify these conclusions, independent mapping of Xyl, Mal, Lac will be necessary.

Four hundred ten segregants were tested both on EMB−lactose and on EMB−xylose. Segregation was always complete; i.e., no cultures were found which had segregated for one factor, and not for the other. This eliminates interpretations based on new segmental losses, or on some forms of autogamy.

It may be argued that the unequal segregations are due to selection. This factor cannot be excluded, but the dominant segregant differs from diploid to diploid. That is to say, other heterozygotes have been isolated from parallel crosses which gave an excess of Lac+ rather than Lac− segregants, or Xyl+ more than Xyl− or both. It seems more likely that the ratios observed are based upon the genetic constitution of the heterozygote.

The Lac+ character of Lac_1 heterozygotes shows that Lac_1+ is dominant to Lac_1−. A number of other loci have been identified, mutations at which lead to the inability to ferment lactose. At two more of these at least, the + allele is dominant. Cultures carrying Lac_2− or Lac_4− were crossed to "*Het*" stocks carrying Lac_1−. A large proportion of the Lac+ prototrophs seen on EMS−lactose turned out to segregate for lactose fermentation. Their variegation, however, was generally periclinal rather than sectorial, with dark, Lac+ centers and light, Lac − margins with only occasional streaks of Lac+. This difference is to be expected, since each of the chromosomes of the diploid carries a Lac− mutation. Therefore, only those segregants in which there has been a crossover between the two Lac loci, bringing the + alleles into coupling phase will be lactose positive. That these cultures are segregating for two Lac− factors has been confirmed by physiological and genetic tests on the segregants.

Heterozygotes from Standard Crosses.—All the heterozygotes so far referred to are the issue of crosses involving the hypothetical "*Het*" factor derived from *H-1*. In these crosses, several per cent of the prototrophs are demonstrably heterozygous. Previous and current controls showed that, if they occurred at all in "normal" crosses, they must be very much rarer. In order to make a more thorough test, advantage was taken of the very close linkage (less than 1% recombination) which has been observed between the Lac_1 and Lac_4 loci mentioned above. $B-M-Lac_1-Lac_4+$ was crossed with $T-L-B_1-Lac_1+Lac_4-$. Much less than 1% of the prototrophs of this cross on EMS−lactose are Lac+. It was reasoned that a Lac+ prototroph might represent either a very rare crossover, or a diploid in which the + factors were carried on opposite chromosomes. Because most haploid recombinants, being Lac−, can be set aside by inspection, this is a fairly efficient way of screening for rare diploids. In this way, heterozygous diploids were also obtained from normal stocks, not carrying "*Het*," but they constitute only about 0.1% of the prototrophs. These prototrophs are generally similar to the previous ones, except that they are somewhat more stable. It has not been established whether new "*Het*" mutations have occurred here. When these diploids are singly heterozygous for various sugar fermentation factors, they also show non-random segregations, but these mutations are not the same as those in the "*Het*" series, so that a direct comparison is not yet possible.

Discussion.—It is not clear what relationship there is between the exceptional persistence of these diploids, and the abnormalities in their segregation. There may be some direct connection between their heteroploidy, i.e., deficiency for the Mal region, and the prolongation of the diplophase. But the very disturbing possibility has not been discounted that these heterozygotes merely reveal a situation which also operates in the formation of haploid prototrophs from transient zygotes. If this is so, the linkage map of *E. coli* K-12 will have to be systematically reexamined, with the use of several unrelated sets of stocks. In any case, the discrepancies affect only the details of chromosome behavior. These heterozygotes, on the other hand, have provided an unexpected confirmation of the sexual basis of genetic recombination in this bacterium.

Although the heteroploidy is somewhat of a limitation, heterozygote formation is a very helpful tool in genetic analysis. Since all types of recombinants, not only prototrophs, can be recovered, it is feasible to extend pedigrees to several generations, and interesting combinations of factors can be put together in a form allowing their use in crosses. For example, a Lac_1- mutation occurring in a $T-L-B_1-$ stock has been transferred to a combination with $B-M-$, allowing it to be compared genetically in crosses with other Lac $-$ mutants induced in $T-L-B_1-$ material.

The experimental production and maintenance of diploids raises questions of dominance and dosage effects. It has already been noted that the normal alleles of several biochemical mutations are dominant, although quantitative comparisons of enzyme competence in heterozygotes, haploids and homozygotes remain to be carried out. The dominance of the type sensitive alleles of genes for phage resistance supports, at least in part, a segregation interpretation for the delayed effects noted by Demerec and Latarjet in induced resistance mutations.[3] On the other hand, for the study of phenotypic lag in bacteria,[4] segregation provides a far larger and more reproducible source of material than mutation.

In respect to lactose fermentation, such a lag cannot last more than a few cell generations in this material, in view of the appearance of numerous Lac $-$ sectors in Lac*v* colonies on EMB $-$ lactose agar. The masking effects noted[5] with yeast asci segregating in the presence of the substrate have thus not beeen found here.

Cytological and single cell studies on these strains are in progress.

Summary.—1. Unstable prototrophs have been isolated from certain crosses in *E. coli* K-12, and characterized as segregating heterozygous diploids.

2. The capacity to produce appreciable numbers of persistent heterozygous diploids is inherited.

3. The segregation of various mutant factors is strongly biased, possibly due to a recessive lethal deletion including a locus affecting maltose fermentation.

4. In the heterozygotes, the type + alleles of factors controlling several fermentations and nutritional requirements are dominant. Sensitivity to bacteriophage T1 is dominant to resistance.

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[1] Tatum, E. L., and Lederberg, J., *J. Bact.*, **53**, 673–684 (1947). Lederberg, J., *Genetics*, **32**, 505–525 (1947).

[2] The composition of EMS-, in grams per liter, is: Sodium Succinate 5; $(NH_4)_2SO_4$ 5; NaCl 1; $MgSO_4$ 1; K_2HPO_4 2; Agar 15; Methylene Blue Hydrochloride 0.065; Eosin Y 0.4. This formula is conveniently stored as a dry mixture of the powdered components.

[3] Demerec, M., and Latarjet, R., *Cold Spring Harbor Symposia Quant. Biol.*, **11**, 38–50 (1946).

[4] Newcombe, H. B., *Genetics*, **33**, 447–476 (1948).

[5] Spiegelman, S., Lindegren, C. C., and Lindegren, G., these PROCEEDINGS, **31**, 95–102 (1945).